L Number	Hits	Search Text	DB	Time stamp
1	2	6284460[pn] and (p near2 '53')	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:38
2	44	array same (p near2 '53')	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:43
3	50	mutation same (p near2 '53')	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:42
4	535	hla near10 class near10 gene	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:43
5	33630	dp or dq or dr near5 beta	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:43
6	548	(dp or dq or dr) near5 beta	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:45
8	32	hla near10 class near10 ((dp or dq or dr) near5 beta)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:44
7	5	array same (hla near10 class near10 gene)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:44
9	5	array same ((dp or dq or dr) near5 beta)	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:44
10	7	((dp or dq or dr) near5 beta) same mutation	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:47
11	151	forensic same array	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:51
12	24	(forensic same array) same polymorph$3	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:49

temperatures (e.g., 20 to 25° C.). When the assay is performed at room temperature, small probes with T_ms of 40° C. or less (e.g., 10 to 20 nt) can provide the discrimination necessary, as shown in the examples below. Probes in this size range are also less likely to fold on themselves under the reaction conditions, an effect that would reduce the binding efficacy of a probe without regard to the structure of the target.

As stated above, the capture probe may interact with the target in any number of ways. For example, in another embodiment, the capture probes may contact more than one region of the target nucleic acid. When the target nucleic acid is folded as described, two or more of the regions that remain single stranded may be sufficiently proximal to allow contact with a single capture probe. The capture oligonucleotide in such a configuration is referred to herein as a "bridge" or "bridging" oligonucleotide, to reflect the fact that it may interact with distal regions within the target nucleic acid. The use of the terms "bridge" and "bridging" is not intended to limit these distal interactions to any particular type of interaction. It is contemplated that these interactions may include non-canonical nucleic acid interactions known in the art, such as G-T base pairs, Hoogstein interactions, triplex structures, quadraplex aggregates, and the multibase hydrogen bonding such as is observed within nucleic acid tertiary structures, such as those found in tRNAs. The terms are also not intended to indicate any particular spatial orientation of the regions of interaction on the target strand, i.e., it is not intended that the order of the contact regions in a bridge oligonucleotide be required to be in the same sequential order as the corresponding contact regions in the target strand. The order may be inverted or otherwise shuffled.

It is known that synthetic oligonucleotides can be hybridized to non-contiguous sequences in both RNA and DNA strands, in a manner that either causes the intervening sequence to loop out, or that bridges the base of an internal folded structure (Richardson et al., J. Am. Chem. Soc., 113:5109 [1991]; Francois et al., Nucl. Acid. Res., 22: 3943 [1994]). However, these references do not suggest the design or use of bridging oligonucleotides that can distinguish between the different folded structures, or that bind with significantly reduced efficiency when the intervening sequence is unstructured. The present invention provides methods for the use and design of bridge capture probes with minimally stable regions of complementarity to make these bridge probes sensitive to changes in the target strand structure. Minimal stability (i.e., with a very low melting temperature), may be created in a number of ways, including by the use of short lengths of complementarity, low G-C basepair content, and/or the use of base analogs or mismatches to reduce the melting temperature. To test the effects of variations in the target structure on the efficiency of capture with different lengths of bridge probes, three test molecules were created; these are shown in schematic representation in FIG. **10**. Test molecule #80 (SEQ ID NO:39) has a long segment of self complementarity and when folded as shown, the 8 basepair hairpin formed by this oligonucleotide is further stabilized by a "tri-loop" sequence in the loop end (i.e., three nucleotides form the loop portion of the hairpin) (Hiraro et al., Nucleic Acids Res. 22(4):576 [1994]). In test molecule #81 (SEQ ID NO:40), the stem is interrupted by 2 mismatches to form a less stable structure, and the region of self-complementarity is entirely removed in test molecule #82 (SEQ ID NO:41). All three of these molecules have identical target regions for the binding of the capture oligonucleotides, and an examination of their use is described in Example 6.

When a bridging oligonucleotide contacts sequences on either side of a basepaired stem, the structure formed is termed a three-way or three-arm junction. Such junctions have been studied extensively to determine their physical structure and to assess the differences that occur in the physical structure when additional nucleotides are included in these structures, When extra nucleotides are included at the junction site, where the three strands come together (i.e., when a 'bulged' structure is formed), it has been shown that the structure is more flexible and that some degree of coaxial stacking between the arms stabilized the structure compared to the unbulged structure (See e.g., Zhong et al., Biochem., 32:6898 [1993]; and Yang et al., Biochem., 35:7959 [1996]). The inclusion of two thymidine nucleotides in the portion of the probe that forms the junction is particularly preferred.

There are a number of approaches that may be used in the design or selection of bridging capture probes. As noted above, the term "capture probes" is not intended to limit the application of the bridging probes of the present invention to the capture of a target strand onto a solid support. Additional applications of the bridging probes are described in the Experimental Examples, below. Furthermore, for simplicity of discussion and to avoid repetition, this section describes one embodiment of the present invention, namely a process for creating bridge oligonucleotides that interact with only two regions of a target nucleic acid. It is not intended, however, that the invention be limited to the use of oligonucleotides that have only two sites of interaction. It is contemplated that bridge oligonucleotides may be created that can interact with many sites on a folded target molecule.

Bridge oligonucleotides may be created by the joining two or more short oligonucleotide sequences. The creation of bridge oligonucleotides may be based upon observations that these sequences have been determined to interact with a given folded target when used in isolation, without limitation to any particular nature of interaction, or they may be deduced to be capable of such interaction by virtue of sequence composition, complementarity, or like analysis. For convenience, such sequences are termed herein "contact sequences," to reflect the putative ability of such a sequence to contact the target molecule. The designation of a particular sequence as a contact sequence is not intended to imply that the sequence is in contact, or is required to contact a target in any particular embodiment.

In alternative embodiments, contact sequences may be joined by synthesizing or otherwise creating a new oligonucleotide that incorporates both sequences into a single molecule. In one embodiment, the sequences are joined contiguously within the bridge oligonucleotide (i.e., without any intervening nucleotides or other space-filling material). In another embodiment, the contact sequences are non-contiguous, with the spacing provided by additional nucleotides. In a preferred embodiment, the contact sequences are bridged by two thymidine nucleotides, as depicted in several of the bridging probes in FIG. **11A**. In another preferred embodiment, the contact sequences in the bridging oligonucleotide are connected by a segment of nucleic acid containing a region of self-complementarity, such that the bridging oligonucleotide itself contains a folded structure. A stem-loop folded structure within the bridge oligonucleotide, if situated opposite a stem in the target nucleic acid, would permit the formation of a four-way Holliday structure, which is stabilized by coaxial stacking of the arms (Duckett el al., Cell 55:79 [1988]).

Alternatively, the bridge oligonucleotide may be created by linking the individual sequences with non-nucleotide spacers such as those commonly known in the art, such as

13	28	(forensic same array) same mutation	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:49
14	200	forensic same (probe or oligonucleotide) same mutation	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:51
15	247	forensic same (probe or oligonucleotide) same polymorph$4	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:51
17	40	(forensic same (probe or oligonucleotide) same polymorph$4) not @ay>1993	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:52
16	9	(forensic same (probe or oligonucleotide) same mutation) not @ay>1993	USPAT; US-PGPUB; EPO; JPO; DERWENT; IBM_TDB	2003/02/25 09:52

0 1' (nucleotide i is forced to be base paired) and 'p 1 0 i-1' (prohibiting nucleotides from 1 to i-1 to be base paired). For example, to generate structures that could be responsible for a major cleavage site at position 90 of HCV1a DNA, folding of 244 nt DNA fragment of HCV1a (FIG. 15) was done using mfold version 2.3 (http://www.ibc.wust1.edu/~zuker) with constraints 'f 90 0 1' and 'p 1 0 89' predicting structure shown in FIG. 16A. It is important that this structure not only predicts a cleavage site at position 90, but also explains cleavages at positions 102–103, 161 and 173, making it a good candidate to represent actual base pairing in the DNA molecule. The structure shown in FIG. 16A does not explain cleavage sites at positions 118–119 and 173. To reveal corresponding structures, the folding was done using constraints 'f 118 0 1' and 'p 1 0 117' (nucleotides 1–117 are not base paired and nucleotide 118 is base paired) with one of resulting structures shown in FIG. 16B. Again this structure not only reasonably predicts cleavage site at position 117–118 but also shows how cleavage at position 123 may happen. The same two structures were identified in the development of the experiments described in Example 8, using manual comparison of the cleavage sites and the 32 suboptimal folds. By either method, the knowledge of the structure specificity of the 5' nuclease made it possible to eliminate from consideration, all predicted structures that would require the cleavage sites to vary from the known substrate structure. This reduced the field of possible structures from 32 to 2. Use of additional enzymes, such as 3' nucleases, or duplex specific chemical agents, that can identify other positions that must be base-paired within a structure can further narrow the field.

In addition to the structural mapping methods described above, there are several methods based on the actions of polymerizing enzymes that may be used to gain structural information. It has long been observed that reverse transcriptases can have difficulty polymerizing through RNA secondary structures. For this reason, reverse transcriptases that can be used at high temperatures have been sought (Myers et al., Biochem., 30:7661 [1991]), in order to facilitate full-length reverse transcription before cloning or PCR amplification. By intentionally using polymerases that produce such pausing effects, structures formed in a template strand may be mapped by the location of the pause sites (e.g., by extension of a labeled primer).

Another approach based on the use of DNA polymerases takes advantage of the observation that some DNA polymerases, upon encountering a fold in the template strand, will apparently polymerize across a structure by a mechanism that has been termed "strand switching," thereby deleting the complement of the structured intermediate sequence. Though an understanding of the mechanism of strand switching is not necessary in order to practice the present invention, it is believed that strand switching involves some degree of displacement synthesis, such that a small portion of a sequence (even to the level of one base), is duplicated, followed by a branch migration that pairs the 3' end of the elongated strand with sequences on the far side of the template structure (Patel et al., Proc. Natl. Acad. Sci. USA 93:2969 [1996]). This mechanism can conceivably be used for structure mapping in at least two ways. For example, if the 3' side of a structure has been mapped using a 3' nuclease in a CFLP® reaction, as discussed above, a primer may be designed such that the 3' end of the primer is poised to polymerize either along or across the structure-forming region. In addition to its template complementary sequence, the primer may be supplied with one or a few degenerate nucleotides (e.g., two or more nucleotides at the same position on different copies of the primer) on the 3' end, to provide opportunity for strand switching, regardless of the downstream sequence. The primer may then be extended under conditions favoring strand switching (Patel et al., supra). The isolation (e.g., by cloning and sequencing) of such sites should identify the sequences that are coming together to form the folded structures, thus facilitating bridge oligonucleotide design. A second approach is similar, but without the use of primers adjacent to any particular putative structure. In this embodiment, a strand to be analyzed is primed using a normal primer, and synthesis is carried out in the same or similar strand switch favoring conditions. The use of conditions that favor base misincorporation (e.g., by the use of manganese in the synthesis reactions), and therefore promote pausing of the polymerase, would provide additional opportunity for branch migration and strand switching. The analysis of the junction sites would then follow as with the first approach. By these methods, both sides of a cleavage structure could be identified. It is also expected that alternative pairing partners for various sequences would be represented in the collection of molecules created.

To distinguish between related nucleic acids, the regions that show different sites of cleavage or modification have the highest probability of having secondary structures that will respond differently to probes in the methods of the present invention. This is for two reasons. First, the cleavage or modification is physical evidence that a structure may form at a given site under the conditions of the cleavage or modification assay. Second, the structures that are detected by the CFLP® method have been found to be predominantly local (i.e., formed from sequences that are close to each other along the nucleic acid strand, Brow et al., supra), so that changes observed are likely to be caused by base changes near the altered cleavage site. By designing oligonucleotide probes to hybridize or complex with the regions showing different sites of cleavage or modification there is a higher probability of finding either a base change (primary structure variation) or a folding change (secondary structure variation) that will affect the complexing of the probe to that site, thus facilitating the distinction between the comparison targets. Because of the complex nature of the folded structure formation as described above and because any given probe may interact with the target in a number of ways, choosing a probe in this way is not a guarantee that any particular probe will provide a diagnostic distinction. This is offered as a guide to increase the probability that it will. When working with an uncharacterized target or set of targets, the use of a multiplicity of such probes will give the most distinctive signature of probe/target complex formation.

In one embodiment, it is preferred that the probes used in the methods of the present invention be short enough to provide distinctive hybridization signatures for variants of a target. Probes longer than about 20 nt (e.g., 20 to 40 nt) can interact with target nucleic acids in a specific manner at elevated temperatures (e.g., higher than about 40° C.) and thus are suitable for use in the present methods. However, probes in this size range may interact with multiple sites on the target if the reaction is performed below about 40° C., reducing the distinction between variants. If this is the case, higher reaction temperatures or more stringent solution conditions (e.g., lower salt, the inclusion of helix-destabilizing agents such as dimethyl sulfoxide or formamide) may prove useful in enhancing the distinction between targets. In a particularly preferred embodiment, the method of the present invention is performed at ambient